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The majority of human breast cancers overexpress cyclin D1. We previously generated mice lacking cyclin D1 using gene targeting. These mice develop essentially normally, revealing that cyclin D1 is dispensable for proliferation of the vast majority of lineages. These findings suggest that targeting of cyclin D1 might be highly specific in shutting off the growth of breast cancer cells, while sparing other tissues. As a first step towards an anti-cyclin D1 therapy, we studied breast cancer susceptibility of cyclin D1-deficient mice. We found that these mice are resistant to breast cancers induced by Neu and Ras oncogenes, while being sensitive to other mammary epithelial oncogenic pathways. Our analyses revealed that in mammary epithelial cells, the Neu-Ras pathway is connected to the cell cycle machinery via cyclin D1, explaining the absolute dependency on cyclin D1 for malignant transformation in mammary glands. Our results suggest that an anti-cyclin D1 therapy might be highly specific in treating human breast cancers with hyperactivated Neu-Ras pathway.

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INTRODUCTION

Several lines of evidence implicate cyclin D1 as a key protein in breast cancer formation. Thus, cyclin D1 gene is amplified in up to 20% of human breast cancers, while cyclin D1 protein is overexpressed in over 50% of these tumors. The overexpression of cyclin D1 is seen in all histologic types of human breast cancers. It can be detected at the earliest stages of breast cancer progression, such as ductal carcinoma in situ, but not in premalignant lesions. Once acquired, cyclin D1 overexpression is maintained in all stages of the disease including the metastatic lesions. Importantly, cyclin D1 overexpression was shown to play a causative role in breast cancer formation (1-4).

We previously generated mice lacking cyclin D1 using gene knockout technology. Surprisingly, we found that these animals develop essentially normally, revealing that cyclin D1 is dispensable for proliferation of the vast majority of cell lineages (5).

The very limited impact of cyclin D1 loss on mouse physiology - together with the well documented role of cyclin D1 overexpression in human breast cancers - suggested to us that the ablation of cyclin D1 might be highly selective in shutting off the proliferation of breast tumor cells while sparing other tissues. **As a first step towards a potential anti-cyclin D1 therapy in human breast cancers, we set to determine whether genetic ablation of cyclin D1 protects mice against breast cancers.**

RESEARCH ACCOMPLISHMENTS

1. Expression of D-type cyclins in normal and cyclin D1$^{-/-}$ mammary glands.

We first determined the expression pattern of D-cyclins in mammary glands of wild-type and cyclin D1$^{-/-}$ females. To this end, we dissected mammary glands from wild-type and cyclin D1-deficient females, prepared protein lysates, and we determined the levels of D-cyclins by the Western blotting. As expected, wild-type mammary glands expressed mostly cyclin

D1, together with low levels of cyclin D2 and D3. Cyclin $D1^{-/-}$ mammary glands lacked cyclin D1, but instead contained modestly elevated levels of cyclin D2 and slightly increased levels of cyclin D3 (Fig. 1c of the enclosed paper by Yu et al.). We presume that these low levels of cyclins D2 and D3 allow normal mammary development in cyclin $D1^{-/-}$ mice.

2. Crosses of cyclin $D1^{-/-}$ mice with MMTV-oncogene mice

We crossed cyclin $D1^{-/-}$ mice with several different strains of breast cancer-prone MMTV-oncogene transgenic mice, and we generated cyclin $D1^{-/-}$/MMTV-oncogene animals. For our studies we employed strains overexpressing v-Ha-Ras , Neu, Myc or Wnt-1 oncogenes. The numbers of mice in each experimental group are shown in Table 1 of the enclosed paper by Yu et al.

3. Appearance of mammary glands

We first compared the appearance of mammary glands of adult, virgin cyclin $D1^{-/-}$/MMTV-oncogene females with that of cyclin $D1^{+/+}$/MMTV-oncogene females. For each of four transgenic strains, we found that the appearance of cyclin $D1^{-/-}$ mammary glands was identical to that of wild-type mice. This is consistent with our earlier observations that cyclin $D1^{-/-}$ mice develop normal mammary glands in a virgin state (5). For our tumor-susceptibility analyses, all females were kept as virgins throughout the entire observation period (except for MMTV-Myc mice).

These control experiments provided us with an additional, unexpected observation. Thus, as reported previously, mammary glands of MMTV-Wnt-1 transgenic mice undergo precocious lobuloalveolar development in a virgin state. As a result, mammary glands of MMTV-Wnt-1 virgin females (and males) resemble that of pregnant wild-type, non-transgenic females (6). Strikingly, we observed the same phenotype in cyclin $D1^{-/-}$/MMTV-Wnt-1 mice (Fig. 1a of the enclosed paper by Yu et al.). This suggests that Wnt-1-dependent proliferative signals do not require cyclin D1. This is in contrast with recent reports that Wnt-1/β-catenin signaling pathway critically impinges on cyclin D1 (7).

4. Breast cancer incidence

We observed MMTV-oncogene mice for breast cancer incidence. We found that the loss of cyclin D1 did not protect cyclin $D1^{-/-}$ mice from breast cancers induced by the Myc and Wnt-1 oncogenes (Fig. 2 and Table 1 of the enclosed paper by Yu et al.). In striking contrast, cyclin $D1^{-/-}$ mice were resistant to breast cancers induced by the Ras and Neu oncogenes. Thus, during the observation period, 19 out of 21 cyclin $D1^{+/+}$/MMTV-Ras mice died of breast cancers, developing a total of 39 tumors, while all 18 cyclin $D1^{-/-}$/MMTV-Ras females remained tumor free (Fig. 2 and Table 1 of the enclosed paper by Yu et al.). Likewise, 26 out of 26 cyclin $D1^{+/+}$/MMTV-Neu animals died of mammary carcinomas, developing a total of 79 tumors, while all 42 cyclin $D1^{-/-}$/MMTV-Neu mice remained healthy and tumor free during the 16-month observation period (Fig. 2 and Table 1 of the enclosed paper by Yu et al.).

5. Examination of the transgene levels in wild-type and cyclin $D1^{-/-}$ mammary glands.

To exclude the possibility that these differences in tumor susceptibility were caused by an inadequate expression of Ras or Neu transgenes in the mammary glands of cyclin $D1^{-/-}$ animals, we determined the transgene RNA levels using reverse transcription-PCR analyses. To this end, we collected mammary glands from cyclin $D1^{+/+}$/MMTV-oncogene and cyclin $D1^{-/-}$/MMTV-oncogene females, isolated RNA, transcribed it into cDNA using the reverse transcriptase, and subjected the cDNA to semi-quantitative PCR amplification. The PCR products were resolved on agarose gels, transferred to membranes and probed with radiolabelled, transgene-specific oligonucleotides. These analyses revealed essentially identical levels of the transgenes in the mammary glands of wild-type and cyclin $D1^{-/-}$ females (Fig. 1b of the enclosed paper by Yu et al.). We concluded that the resistance of cyclin $D1^{-/-}$ mammary glands to Ras- and Neu-driven transformation was not caused by the absence of transgene expression in cyclin $D1^{-/-}$ mammary glands. Instead, we concluded that cyclin D1 is critically required for Ras- and Neu-induced mammary tumorigenesis, and consequently, the loss of cyclin D1 renders cyclin $D1^{-/-}$ mice resistant to breast cancers induced by these two oncogenes.

6. Crosses between MMTV-oncogene mice and cyclin D2$^{-/-}$ and cyclin D3$^{-/-}$ mice

To determine whether this critical role for cyclin D in Ras- and Neu-driven breast tumorigenesis is uniquely associated with cyclin D1, we crossed mice lacking either of the other two members of the D-cyclins family, namely cyclin D2 or cyclin D3 (which we previously generated) with MMTV-Ras mice. We found that cyclin D2$^{-/-}$/MMTV-Ras and cyclin D3$^{-/-}$ /MMTV-Ras mice were susceptible to breast cancers (Table 1 of the enclosed paper by Yu et al.), pointing to a unique role for cyclin D1 in Ras-induced breast tumorigenesis.

7. Analyses of the estrogen receptor levels (ER)

We hypothesized that this specific requirement for cyclin D1 in Ras- and Neu-driven but not in Myc- and Wnt-1 driven tumorigenesis might reflect the estrogen receptor (ER) status of these tumors. Cyclin D1 was shown to act as an ER co-activator in vitro (8,9), and we hypothesized that tumors dependent on cyclin D1 (Ras- and Neu-driven) might be ER positive. To address this hypothesis, we performed the following analyses:

7a. We isolated mRNA from Ras-, Neu-, Myc- and Wnt-1-driven tumors arising in cyclin D1$^{+/+}$ mice and we determined the levels of ER transcripts by Northern blotting. These analyses revealed extremely low, essentially negligible levels of the ER mRNA in all tumors, irrespective of the expressed transgene.

7b. We prepared protein lysates from Ras-, Neu-, Myc- and Wnt-1-driven tumors arising in cyclin D1$^{+/+}$ mice and we determined the levels of ER by Western blotting. These analyses revealed extremely low levels of the ER in all tumors, irrespective of the expressed transgene.

7c. We established primary cultures of breast cancer cells from tumors arising in cyclin D1$^{+/+}$MMTV-Ras, MMTV-Neu, MMTV-Wnt-1 and MMTV-Myc mice. We determined estrogen-dependence of these cells for proliferation by treating them with estradiol or tamoxifen. For control we used MCF-7 cell line. In contrast to MCF-7 cells, MMTV-oncogene breast

cancer cell proliferation was not stimulated by estradiol and was not inhibited by tamoxifen.

7d. We ovariectomized three cyclin D1$^{+/+}$/MMTV-Ras females at 3 weeks of age and we determined the impact of this procedure on tumor occurrence. We found that ovariectomy did not prevent tumor occurrence.

7e. We ovariectomized three cyclin D1$^{+/+}$/MMTV-Ras, MMTV-Neu, MMTV-Myc and MMTV-Wnt-1 females as soon as breast tumor was detected and we determined the impact of this procedure on tumor growth. We found that ovariectomy did not slow down tumor growth.

We concluded that the specific requirement for cyclin D1 in Ras- and Neu-driven tumors cannot be explained by the ER status of these cancers.

8. Expression of D-cyclins in breast tumors

To further probe the molecular basis of this strict requirement for cyclin D1 in Ras- and Neu-induced, but not in Wnt-1- and Myc-driven mammary tumorigenesis, we examined the expression pattern of the three D-type cyclins in breast tumors arising in cyclin D1$^{+/+}$ transgenic females. These analyses revealed that breast tumors arising in MMTV-Ras and MMTV-Neu mice expressed virtually only cyclin D1 (no cyclin D2, only trace levels of cyclin D3), see Fig. 3a and Fig. 3c, first lane of the enclosed paper by Yu et al. In striking contrast, tumors arising in MMTV-Wnt-1 and MMTV-Myc females expressed - in addition to cyclin D1 - also high levels of cyclin D2 (Fig. 3a of the enclosed paper by Yu et al). Importantly, we verified that all tumors included into analyses arose from luminal epithelial cells, as they expressed keratin 19 (data not shown). These findings suggested to us that in mammary epithelial cells, Ras and Neu oncogenes communicate with the cell cycle machinery via cyclin D1, while Wnt-1 and Myc can signal via other targets.

9. Expression of D-cyclins in breast cancer cell lines

We further extended these analyses by studying pure populations of mouse breast cancer cells grown in vitro. Thus, we compared the expression pattern of D-cyclins between a non-transformed mouse mammary

epithelial cell line, HC11, a breast cancer cell line SH1.1 derived from a tumor arising in MMTV-v-Ha-Ras female mouse, and a breast cancer cell line 13Ma1a derived from a tumor arising in MMTV-c-Myc female mouse (the two transgenic strains used as a source of tumor cells are the same as those used in our tumor-susceptibility analyses). As expected, non-transformed mammary epithelial cells expressed cyclin D1 as a sole D-cyclin (Fig. 3b of the enclosed paper by Yu et al.), a characteristic feature of mammary epithelial luminal cells. Ras-transformed breast cancer cells displayed grossly elevated levels of cyclin D1, but did not express cyclin D2. In contrast, Myc-transformed breast cancer cells contained high levels of cyclin D2 in addition to cyclin D1 (Fig. 3b of the enclosed paper by Yu et al.). Importantly, we verified that the Myc-expressing breast cancer cell line 13Ma1a arose from luminal epithelial cells, as it expressed keratin 19 (data not shown). The results of this experiment strongly support our interpretation that the Ras oncogene (and the Neu oncogene which is known to operate upstream of Ras) signal in mammary epithelial cells by inducing exclusively cyclin D1, while Myc oncogene can act via other targets in these cells.

10. Generation of cyclin D2-->D1 mice (Aim 2).

Cyclin D2-->D1 gene targeting construct was prepared as described in last year's report and electroporated into embryonal stem (ES) cells. We encountered very low homologous recombination rate (less that 1%) and we had to perform four electroporations in order to obtain four ES cell clones in which homologous recombination took place. These four clones have been extensively checked by Southern blotting for single integration sites, proper incorporation at the 3' and 5' ends etc. They were also karyotyped. Injections of these clones into blastocysts of C57BL/6 mice have been delayed, because the infection of our mouse facility with the mouse hepatitis virus (MHV) prohibited us from performing blastocyst injections for approx. nine months.

Our colony is now MHV-free and we resumed blastocyst injections. We are now injecting cyclin D2-->D1 ES cells into blastocysts. We expect to obtain the germline transmission before the end of 2001. We plan to analyze cyclin D2-->D1 mice in 2002.

KEY RESEARCH ACCOMPLISHMENTS

Demonstration that Ras and Neu oncogenes are connected to the cell cycle machinery in mammary epithelial cells exclusively through cyclin D1. These findings suggests a novel, potentially highly specific therapy for breast cancers, centered around cyclin D1.

REPORTABLE OUTCOMES

1. Publications

Q. Yu, Y. Geng, P. Sicinski. Specific protection against breast cancers by cyclin D1 ablation. Nature, 411, 1017-1021 (2001).

2. Presentations (by P. Sicinski)

Brigham and Women's Hospital, Boston, USA (February 2001)
Max-Planck Institut, Munich, Germany (May, 2001)
Harvard Medical School, Boston, USA (June 2001)
Imperial Cancer Research Fund, London, UK (June 2001)
Chester Beatty Laboratories, London, UK (June 2001).

CONCLUSIONS

Cyclin D1 protein was shown to play important role in breast cancer formation. In the research described above we set to determine whether inactivation of cyclin D1 would protect mice against breast cancers. Our analyses revealed that mice lacking cyclin D1 are completely immune to certain breast cancers. These studies suggest a possibility of a novel, highly specific therapy for human breast cancers, centered around cyclin D1. Thus, our work strongly suggests that anti-cyclin D1 therapy might be highly selective in shutting off the growth of human breast cancers while sparing other tissues.

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Genetically altered mice immune to breast cancer

June 27, 2001 Posted: 2:01 PM EDT (1801 GMT)

INDIANAPOLIS, Indiana (AP) -- Scientists have genetically engineered mice that are immune to some of the most common types of breast cancer, a development they say brings science a step closer to creating drugs to precisely block the spread of breast cancer in humans.

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The research involves a protein linked to half of all human breast cancers. The bioengineered mice lack that protein, which tumors need to grow.

Though it could take years to develop a drug therapy targeting the protein, the findings are dramatic proof that certain breast cancers can occur only when the protein is present, said Christopher Widnell, scientific program director of the Atlanta-based American Cancer Society.

"This work is taking us to the next phase, where you can actually start designing intelligent treatments for individual tumors," said Widnell, who wasn't involved in the research.

The Dana-Farber Cancer Institute researchers produced the cancer-resistant mice by building on earlier success in engineering mice that don't express the protein cyclin D1, one of many proteins that regulate cell growth.

They wanted to test whether eliminating the protein in mice prone to certain breast cancers could keep them cancer-free.

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The researchers bred the mice engineered to not express cyclin D1 with four other types of laboratory mice, each prone to different types of breast cancer, and monitored their offspring for signs of cancer. Two of the resulting cross-breeds were immune to the type of breast cancer for which they carried a gene.

Signals from cancer genes

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Piotr Sicinski, a Dana-Farber researcher, said the findings clearly show two cancer genes called Neu and Ras can only turn normal cells into cancer cells by sending signals through the cyclin D1 protein.

The fact the mice with the two other cancer genes, called Wnt-1 and Myc, developed breast cancers means those cancer genes are capable of signaling through other cell-regulating proteins, Sicinski said.

The research appears in Thursday's issue of the journal Nature.

Sicinski and his colleagues want to see other researchers try to target cyclin D1 using existing cancer-blocking drugs.

He suggested pairing drugs that target a protein partner of the cell-regulating proteins that include cyclin D1 with Genentech's breast cancer drug, Herceptin, which aims to block the Neu cancer gene. That cancer gene has been linked to about 30 percent of human breast cancers.

"If you had a way to take out cyclin D1 you could completely unplug the (cancer gene) pathways from the cell cycle machinery without compromising the patient's health," Sicinski said.

In an accompanying Nature commentary, two Danish scientists said that when more is known about the cellular changes that the Neu and Ras cancer genes induce through cyclin D1, physicians might be able to create molecular profiles of breast cancer patients to target their unique mix of tumors.

"It might one day be possible to provide tailor-made treatments," say Jiri Bartek and Jiri Lukas of the Institute of Cancer Biology, Danish Cancer Society, Copenhagen.

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Researchers able to genetically alter mice resistant to common breast cancers

Associated Press

Scientists have created mice that are immune to some of the most common types of breast cancer, a development they say brings science a step closer to developing drugs to precisely block the spread of breast cancer in humans.

The research involves a protein linked to half of all human breast cancers. The bioengineered mice lack that protein, which some tumors need to grow.

Though it could take years to develop a drug therapy targeting the protein, the findings are dramatic proof that certain breast cancers can occur only when the protein is present, said Christopher Widnell, scientific program director of the Atlanta-based American Cancer Society.

"This work is taking us to the next phase, where you can actually start designing intelligent treatments for individual tumors," said Widnell, who wasn't involved in the research.

The Dana-Farber Cancer Institute scientists who conducted the research said they hope their findings will inspire more research to target the protein using existing cancer-blocking drugs.

They produced the cancer-resistant mice by building on earlier success in engineering mice that don't express the protein cyclin D1, one of many proteins that regulate cell growth. Because cyclin D1 is found in abnormally high amounts in half of human breast cancers, it has become the focus of much scientific scrutiny.

The Dana-Farber scientists wanted to test whether eliminating the protein in mice prone to certain breast cancers could keep them cancer-free.

The researchers bred the mice engineered to not express cyclin D1 with four other types of laboratory mice, each prone to different types of breast cancer, and monitored their offspring for signs of cancer. Two of the resulting cross-breeds were immune to the type of breast cancer for which they carried a gene.

Piotr Sicinski, a Dana-Farber researcher, said the findings clearly show two cancer genes called Neu and Ras can only turn normal cells into cancer cells by sending signals through the cyclin D1 protein.

The fact the mice with the two other cancer genes, called Wnt-1 and Myc, developed breast cancers means those cancer genes are capable of signaling though other cell-regulating proteins, Sicinski said.

The research appears in Thursday's issue of the journal Nature.

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In an accompanying Nature commentary, two Danish scientists said that when more is known about the cellular changes that the Neu and Ras cancer genes induce through cyclin D1, physicians might be able to create molecular profiles of breast cancer patients to target their unique mix of tumors.

"It might one day be possible to provide tailor-made treatments," say Jiri Bartek and Jiri Lukas of the Institute of Cancer Biology, Danish Cancer Society, Copenhagen.

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Specific protection against breast cancers by cyclin D1 ablation

Qunyan Yu, Yan Geng & Piotr Sicinski

Department of Cancer Biology, Dana-Farber Cancer Institute, and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, USA

Breast cancer is the most common malignancy among women. Most of these cancers overexpress cyclin D1, a component of the core cell-cycle machinery. We previously generated mice lacking cyclin D1 using gene targeting. Here we report that these cyclin D1-deficient mice are resistant to breast cancers induced by the *neu* and *ras* oncogenes. However, animals lacking cyclin D1 remain fully sensitive to other oncogenic pathways of the mammary epithelium, such as those driven by c-*myc* or *Wnt-1*. Our analyses revealed that, in mammary epithelial cells, the Neu–Ras pathway is connected to the cell-cycle machinery by cyclin D1, explaining the absolute dependency on cyclin D1 for malignant transformation in this tissue. Our results suggest that an anti-cyclin D1 therapy might be highly specific in treating human breast cancers with activated Neu–Ras pathways.

Cyclin D1 belongs to the family of three closely related D-type cyclins, termed cyclin D1, D2 and D3. These three proteins are expressed in an overlapping, redundant fashion in all proliferating cell types. D-cyclins collectively control cell-cycle progression by activating their cyclin-dependent kinase partners, CDK4 and CDK6, which leads to phosphorylation of the retinoblastoma protein, and in turn to the advance through the G1 phase of the cell cycle[1].

Several lines of evidence point to an important role for cyclin D1 in breast cancer formation. The *cyclin D1* gene is amplified in up to 20% of human breast cancers[2], while cyclin D1 protein is overexpressed in over 50% of human mammary carcinomas[3–5]. The overexpression of cyclin D1 is seen in all histological types of human breast cancers[3]. It can be detected at the earliest stages of breast cancer progression, such as ductal carcinoma *in situ*, but not in premalignant lesions[6]. Once acquired, overexpression of cyclin D1 is maintained in all stages of the disease including the metastatic lesions[3,7]. Importantly, overexpression of cyclin D1 seems to have a causative role in breast cancer formation, as transgenic mice engineered to overexpress cyclin D1 in mammary glands succumb to breast cancers[8].

We and others previously generated cyclin D1-deficient mice[9,10]. We found that these *cyclin D1*$^{-/-}$ animals were viable and showed a narrow set of developmental abnormalities restricted to the retina and the nervous system[9,10]. In adult mice, however, loss of cyclin D1 had virtually no impact on mouse physiology, except that the mammary glands of *cyclin D1*$^{-/-}$ females failed to undergo full lobuloalveolar development during the late stage of pregnancy. This defect was restricted to pregnancy-associated proliferation, because *cyclin D1*$^{-/-}$ mice developed normal mammary glands during sexual maturation[9,10].

The limited impact of cyclin D1 loss on mouse physiology—together with the well documented role of cyclin D1 overexpression in human breast cancers—suggested to us that the ablation of cyclin D1 might be highly selective in shutting off the proliferation of breast-tumour cells while sparing other tissues. As a first step towards a potential strategy for anti-cyclin D1 therapy in human breast cancers, we investigated whether the ablation of cyclin D1 protects *cyclin D1*$^{-/-}$ mice against breast cancers.

To address this possibility, we crossed *cyclin D1*$^{-/-}$ mice with four different strains of breast-cancer-prone mouse mammary tumour virus (MMTV)-oncogene transgenic mice, and we generated *cyclin D1*$^{-/-}$/MMTV-oncogene animals. For our studies we used strains overexpressing the oncogenes v-Ha-*ras* (ref. 11), c-*neu* (ref. 12), c-*myc* (ref. 13) and *Wnt-1* (ref. 14).

Analyses of mammary glands

We started our analyses by determining the expression pattern of D-cyclins in mammary glands of non-transgenic, virgin wild-type and *cyclin D1*$^{-/-}$ females. Wild-type mammary glands expressed mostly cyclin D1, together with low levels of cyclin D2 and D3. Mammary glands of *cyclin D1*$^{-/-}$ females lacked cyclin D1, but instead contained modestly elevated levels of cyclin D2 and slightly increased levels of cyclin D3 (Fig. 1c). We presume that these low levels of cyclins D2 and D3 allow normal mammary development in *cyclin D1*$^{-/-}$ virgin mice.

We next compared the appearance of mammary glands of adult, virgin *cyclin D1*$^{-/-}$/MMTV-oncogene females with that of *cyclin D1*$^{+/+}$/MMTV-oncogene females. For each of four transgenic strains, we found that the appearance of *cyclin D1*$^{-/-}$ mammary glands was identical to that of wild-type mice (Fig. 1a). This is consistent with our earlier observations that *cyclin D1*$^{-/-}$ mice develop normal mammary glands in a virgin state[9]. For our tumour-susceptibility analyses, all females were kept as virgins throughout the entire observation period, except for the MMTV-*myc* mice (see Methods).

These control experiments provided us with an additional, unexpected observation. As reported previously[14], mammary glands of MMTV-*Wnt-1* transgenic mice undergo precocious lobuloalveolar development in a virgin state. As a result, mammary glands of MMTV-*Wnt-1* virgin females (and males) resemble those of pregnant wild-type, non-transgenic females[14]. Strikingly, we observed the same phenotype in *cyclin D1*$^{-/-}$/MMTV-*Wnt-1* mice (Fig. 1a). This suggests that Wnt-1-dependent proliferative signals do not require cyclin D1. This is in contrast with recent reports that the Wnt-1–β-catenin signalling pathway critically impinges on cyclin D1 (refs 15, 16). It also reveals that *cyclin D1*$^{-/-}$ mammary epithelium can undergo lobuloalveolar development under certain conditions.

Incidence of breast cancer

We observed MMTV-oncogene mice for breast cancer incidence. We found that the loss of cyclin D1 did not protect *cyclin D1*$^{-/-}$mice from breast cancers induced by the *myc* and *Wnt-1* oncogenes (Fig. 2 and Table 1). In marked contrast, *cyclin D1*$^{-/-}$ mice were resistant to breast cancers induced by the *ras* and *neu* oncogenes. Thus, during the observation period, 19 of 21 *cyclin D1*$^{+/+}$/MMTV-*ras* mice died of breast cancers, developing a total of 39 tumours, whereas all 18 *cyclin D1*$^{-/-}$/MMTV-*ras* females remained free of tumours (Fig. 2 and Table 1). Likewise, 26 of 26 *cyclin D1*$^{+/+}$/MMTV-*neu* animals died of mammary carcinomas, developing a total of 79 tumours,

Table 1 Incidence of mammary carcinomas in transgenic mice

	D1$^{-/-}$/MMTV	*D1*$^{+/+}$/MMTV	*D1*$^{-/-}$	Wild type	*D2*$^{-/-}$/MMTV	*D3*$^{-/-}$/MMTV
MMTV-*v-Ha-ras*	0/18 (0)	19/21 (39)			6/11 (20)	8/13 (25)
MMTV-*c-neu*	0/42 (0)	26/26 (79)				
MMTV-*c-myc*	12/20 (18)	16/22 (21)				
MMTV-*Wnt-1*	15/16 (28)	20/20 (38)				
Non-transgenic			0/27 (0)	0/20 (0)		

The observation period was 70 weeks for MMTV-c-*neu*, MMTV-c-*myc* transgenic mice and for non-transgenic wild-type and *cyclin D1*$^{-/-}$ controls, and 50 weeks for MMTV-*ras* and MMTV-*Wnt-1* animals. The ratios of females displaying mammary carcinomas to the total number of observed females are shown, along with total number of breast tumours observed among females of each genotype (in parentheses).

whereas all 42 *cyclin D1*$^{-/-}$/MMTV-*neu* mice remained healthy and free of tumours during the observation period (Fig. 2 and Table 1).

The possibility that these differences were caused by an inadequate expression of *ras* or *neu* transgenes in the mammary glands of *cyclin D1*$^{-/-}$ animals was ruled out by the reverse transcription with polymerase chain reaction (PCR) analyses. These analyses revealed similar levels of the transgenes in the mammary glands of wild-type and *cyclin D1*$^{-/-}$ females (Fig. 1b). We concluded that cyclin D1 is critically required for Ras- and Neu-induced mammary tumorigenesis, and, consequently, the loss of cyclin D1 renders *cyclin D1*$^{-/-}$ mice resistant to breast cancers induced by these two oncogenes.

To determine whether this critical role is uniquely associated with cyclin D1, we crossed mice lacking either of the other two members of the D-cyclin family, namely cyclin D2 (ref. 17) and cyclin D3 (E. Sicinska and P.S., in preparation) with MMTV-*ras* mice. We found that *cyclin D2*$^{-/-}$/MMTV-*ras* and *cyclin D3*$^{-/-}$/MMTV-*ras* mice were susceptible to breast cancers (Table 1), pointing to a unique role for cyclin D1 in Ras-induced breast tumorigenesis.

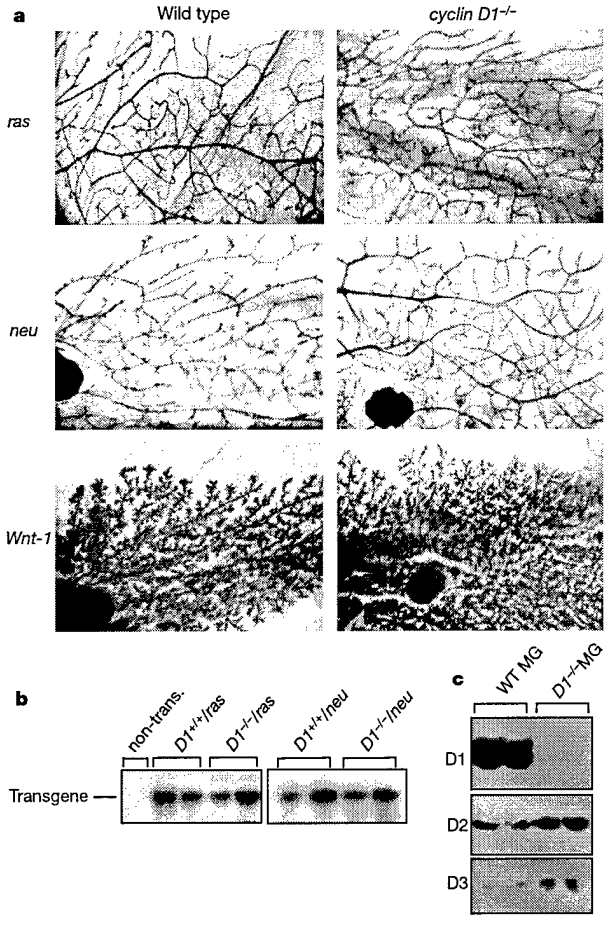

Figure 1 Analyses of mammary glands. **a**, Appearance of mammary glands in *cyclin D1* wild-type/MMTV-oncogene and *cyclin D1*$^{-/-}$/MMTV-oncogene virgin females. Mammary epithelial whole mounts were stained with carmine red. Magnification ~3×. **b**, Expression of the transgenes in wild-type (*cyclin D1*$^{+/+}$) and *cyclin D1*$^{-/-}$ mammary glands (see Methods). non-trans., non-transgenic control; *D1*$^{+/+}$/*ras*, *cyclin D1*$^{+/+}$/MMTV-v-Ha-*ras*; *D1*$^{-/-}$/*ras*, *cyclinD1*$^{-/-}$/MMTV-v-Ha-*ras*; *D1*$^{+/+}$/*neu*, *cyclinD1*$^{+/+}$/MMTV-c-*neu*; *D1*$^{-/-}$/*neu*, *cyclin D1*$^{-/-}$/MMTV-c-*neu*. **c**, Expression of cyclins D1, D2 and D3 in mammary glands of virgin wild-type (WT MG) and *cyclin D1*$^{-/-}$ female mice (*D1*$^{-/-}$MG), as detected by western blotting.

Expression of D-cyclins in breast tumours

To understand the molecular basis of this strict requirement for cyclin D1 in Ras- and Neu-induced, but not in Wnt-1- and Myc-driven, mammary tumorigenesis, we examined the expression pattern of the three D-type cyclins in breast tumours arising in *cyclin D1*$^{+/+}$ transgenic females. These analyses revealed that breast tumours arising in MMTV-*ras* and MMTV-*neu* mice expressed virtually only cyclin D1 (no cyclin D2, only very low levels of cyclin D3; Fig. 3a and c, first lane). In contrast, several tumours arising in MMTV-*Wnt-1* and MMTV-*myc* females expressed, in addition to cyclin D1, also high levels of D2 (Fig. 3a). Importantly, we verified that all tumours included in analyses arose from luminal epithelial cells, as they expressed keratin 19 (data not shown). These findings indicate that, in mammary epithelial cells, *ras* and *neu* oncogenes communicate with the cell-cycle machinery through cyclin D1, whereas *Wnt-1* and *myc* can signal through other targets.

We extended these analyses by studying pure populations of mouse breast cancer cells grown *in vitro*. We compared the expression pattern of D-cyclins between a non-transformed mouse mammary epithelial cell line, HC11 (ref. 18), a breast-cancer cell line, SH1.1, derived from a tumour arising in a MMTV-v-Ha-*ras* female mouse, and a breast cancer cell line, 13Ma1a, derived from a tumour arising in a MMTV-c-*myc* female mouse (the two transgenic strains used as a source of tumour cells are the same as those used in our tumour-susceptibility analyses). As expected[19], non-transformed mammary epithelial cells expressed cyclin D1 as the major D-cyclin (Fig. 3b). *ras*-transformed breast cancer cells displayed grossly elevated levels of cyclin D1, but did not express cyclin D2. In contrast, *myc*-transformed breast cancer cells contained high levels of cyclin D2 in addition to cyclin D1 (Fig. 3b). Importantly, we verified that the Myc-expressing breast cancer cell line, 13Ma1a, arose from luminal epithelial cells, as it expressed keratin 19 (data not shown). The results of this experiment strongly support our interpretation that the *ras* oncogene (and the *neu* oncogene, which is known to operate upstream of *ras* (ref. 20) signals in mammary epithelial cells by inducing exclusively cyclin D1, whereas the *myc* oncogene can act through other targets in these cells.

Ras and Neu action in other cell types

We next asked whether the absolute requirement for cyclin D1 in Ras- and Neu-driven tumorigenesis operated in all cell types of a body. Our analyses of MMTV-oncogene females indicated that this was not true. Thus, one of the four transgenic strains used for our analyses, that is MMTV-*ras* mice, succumb to salivary gland

tumours, albeit with a very low frequency, in addition to high-frequency mammary carcinomas[11]. We observed one salivary carcinoma among 21 *cyclin D1*$^{+/+}$/MMTV-*ras* females (not shown), with 19 of the mice displaying breast tumours. We also observed three salivary adenocarcinomas among *cyclin D1*$^{-/-}$/MMTV-*ras* mice (not shown), while none of these mice developed breast tumours. Hence, these *cyclin D1*$^{-/-}$/MMTV-*ras* mice are susceptible to Ras-induced salivary tumours in the absence of cyclin D1.

To explore this idea further, we infected wild-type and *cyclin D1*$^{-/-}$ 3T3 mouse embryo fibroblasts with retroviruses encoding activated *ras* or *neu* oncogenes, and scored the ability of these cells to grow in soft agar and to form tumours in nude mice. These analyses revealed that *cyclin D1*$^{-/-}$ fibroblasts became fully transformed by *ras* and *neu* oncogenes, grew vigorously in soft agar (Fig. 4a) and formed tumours in nude mice (Fig 4b). Hence, in contrast to mammary epithelial cells, in fibroblasts *ras* and *neu* oncogenes are able to drive malignant transformation in the absence of cyclin D1.

To understand this difference between mammary epithelial cells and fibroblasts, we examined the expression of D-cyclins in tumours formed by *ras*- and *neu*-transformed fibroblasts injected into nude mice, and compared it with the pattern observed in mammary epithelial tumours. As described above, mammary tumours induced by Ras or Neu expressed cyclin D1 as the major D-type cyclin (Fig. 3c, lane 1). In contrast, Ras- and Neu-driven fibroblast tumours expressed high levels of cyclin D2 and D3 in addition to cyclin D1 (Fig. 3c, lanes 2–6). We conclude that—unlike in mammary epithelial cells—*ras* and *neu* oncogenes in fibroblasts can communicate with the cell-cycle machinery also through cyclins D2 and D3. Consequently, *ras* and *neu* oncogenes continue to elicit malignant transformation even in the absence of cyclin D1. We presume that the same is true in other cell types where cyclin D1 was shown to be dispensable for transformation by *ras*. However, our analyses do not allow us to distinguish whether the induction itself, or simply the presence of particular combinations of D-cyclins, is required for malignant transformation of different cell types by oncogenic Ras and Neu.

Discussion

The *ras* oncogene, acting through the mitogen-activated protein kinase pathway, is known to induce cyclin D1 by acting on cyclin D1 promoter[21–24]. Similarly, the *neu* oncogene, which is known to operate upstream of *ras*, upregulates the promoter of the *cyclin D1* gene[25]. Our analyses of *cyclin D1*$^{-/-}$ mice revealed that the *ras* and *neu* oncogenes are absolutely dependent on cyclin D1 for malignant transformation of mammary glands. Moreover, we found that in mammary epithelial cells, activation of *ras* and *neu* oncogenes leads to induction of cyclin D1 messenger RNA, but not mRNA of cyclin D2 or cyclin D3. We conclude that in this particular cell type, *ras* and *neu* oncogenes are connected to the cell-cycle machinery exclusively through the promoter of the *cyclin D1* gene, explaining the absolute dependency of these two oncogenes on cyclin D1.

This conclusion is strengthened by our analyses of cyclin E→D1 'knock-in' mice that we generated[26]. In these knock-in animals, the coding sequences of the *cyclin D1* gene have been deleted and replaced by that of human *cyclin E*. In the tissues of cyclin E→D1 mice the expression of human cyclin E is driven by the cyclin D1 promoter, and it closely mimics the expression pattern of cyclin D1 in wild-type mice[26]. We crossed cyclin E→D1 mice with an MMTV-*neu* strain and generated cyclin E→D1/MMTV-*neu* females. These females, lacking cyclin D1, displayed an incidence of breast cancer similar to that of *cyclin D1*$^{+/+}$/MMTV-*neu* animals (data not shown). Notably, we found that breast cancers arising in cyclin E→D1/MMTV-*neu* females expressed high levels of human cyclin E mRNA and protein (data not shown). These findings further confirm that the Neu–Ras pathway acts through regulatory elements located within the cyclin D1 promoter. They also reveal that cyclin E can replace cyclin D1 not only in mouse development—as was shown before[26]—but also in driving proliferation of breast cancer cells.

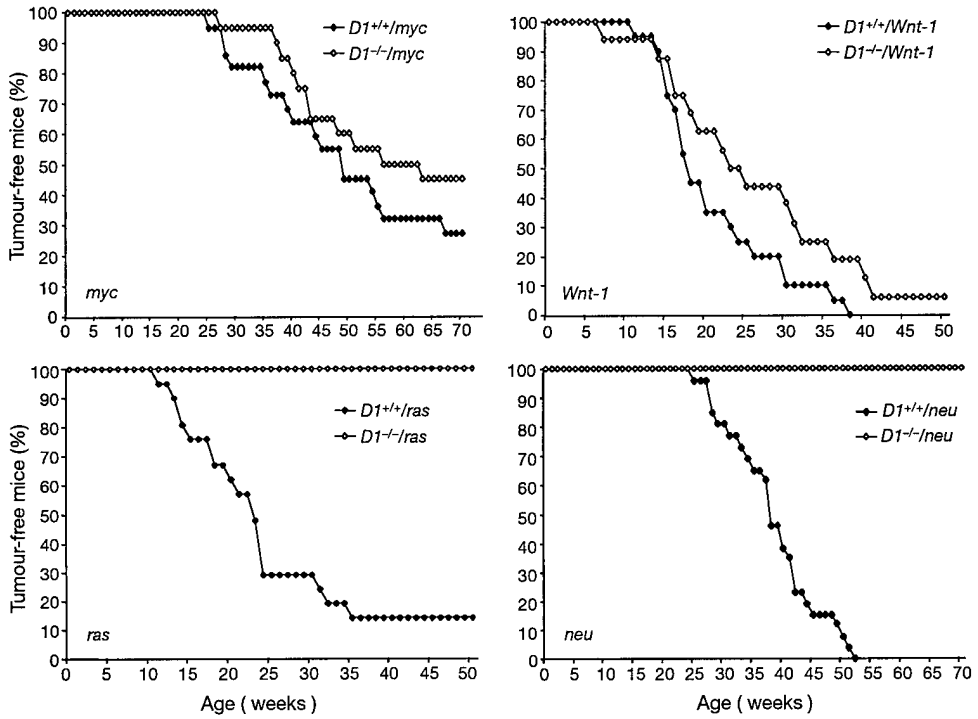

Figure 2 The occurrence of breast cancers in *cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ transgenic mice. *myc*, MMTV-c-*myc* transgene; *Wnt-1*, MMTV-*Wnt-1* transgene, *ras*, MMTV-v-Ha-*ras* transgene, *neu*, MMTV-c-*neu* transgene.

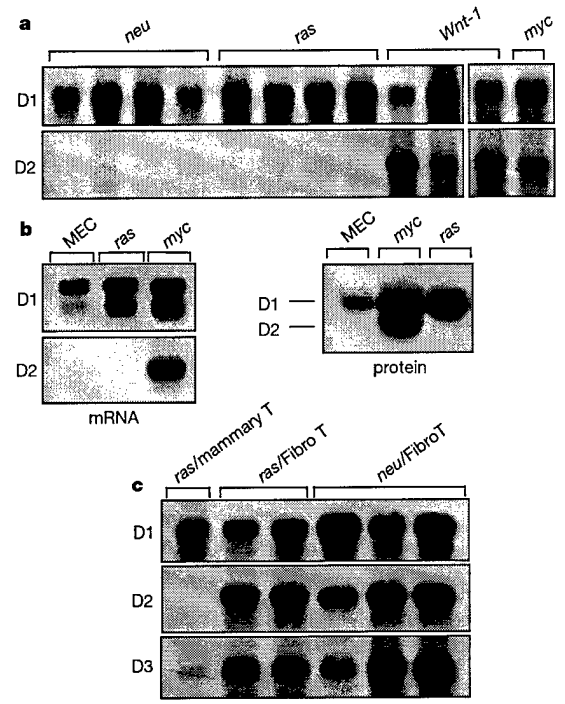

Figure 3 Levels of D-cyclins in tumours. **a**, Expression of cyclins D1 and D2 in tumours arising in MMTV-c-*neu*, MMTV-v-Ha-*ras*, MMTV-*Wnt-1* and MMTV-c-*myc* transgenic females, as detected by northern blotting. **b**, Expression of cyclins D1 and D2 in a non-transformed mouse mammary epithelial cell line, HC11 (MEC), in breast cancer cell line SH1.1 (derived from a MMTV-v-Ha-*ras* transgenic mouse) (*ras*), and in breast cancer cell line 13Ma1a (derived from a MMTV-c-*myc* transgenic mouse) (*myc*), as detected by northern blotting (left panel) and western blotting (right panel). **c**, Expression of D-cyclins in tumours formed by *ras*- (*ras*/Fibro T) and *neu*-transformed fibroblasts (*neu*/Fibro T) injected into nude mice, as detected by northern blotting. Result from a mammary tumour deriving from a MMTV-v-Ha-*ras* mouse (*ras*/Mammary T) is presented in lane 1 for comparison.

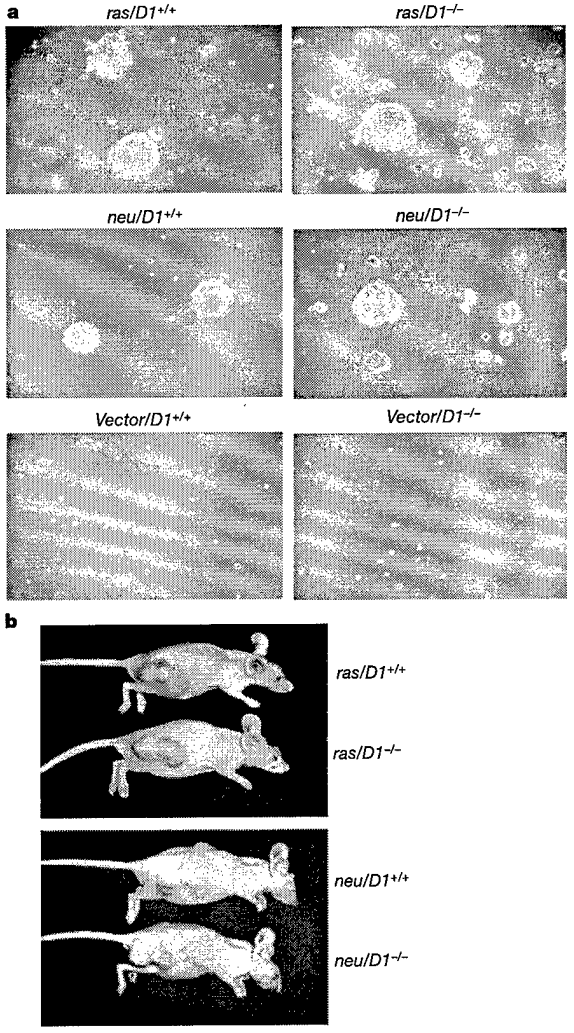

Figure 4 Malignant transformation of *cyclin D1*$^{-/-}$ cells by Ras and Neu. **a**, Wild-type (*cyclin D1*$^{+/+}$) and *cyclin D1*$^{-/-}$ 3T3 mouse embryo fibroblasts were infected with retroviruses encoding activated *ras* or *neu* oncogenes or with a control (empty) vector, and the ability of cells to form colonies in soft agar was scored. The photographs were taken after 13 days. Magnification ~15×. **b**, The cells from **a** were injected into nude mice, and the ability to form tumours was scored. Animals were photographed 3 weeks (*ras*) or 4 weeks (*neu*) after the injection. Cells infected with an empty vector were injected into the contralateral flanks and did not form tumours (not shown).

In contrast to Ras- and Neu-induced carcinogenesis, our analyses revealed that *myc* and *Wnt-1* oncogenes can communicate with the cell-cycle machinery in mammary epithelial cells through targets other than cyclin D1. Indeed, the *myc* oncogene may be connected to the cell-cycle machinery through targets acting downstream of D-cyclins[27,28]. Consequently, Myc (as well as Wnt-1) continue to elicit malignant transformation even in the absence of cyclin D1.

In clear contrast to mammary epithelial cells, we found that in other cell types *ras* and *neu* oncogenes continued to elicit malignant transformation even in the absence of cyclin D1. Robles *et al.* showed that *cyclin D1*$^{-/-}$ mice remain susceptible to Ras-driven skin papillomas, although the number of skin tumours per mouse was lower in *cyclin D1*$^{-/-}$ mice than in *cyclin D1*$^{+/+}$ animals[29]. Our analyses suggest that, in cell types other than mammary epithelium, *ras* and *neu* oncogenes can also signal through cyclins D2 and D3. These results challenge the notion that the wiring of oncogenic pathways to the cell-cycle machinery operates in the same fashion in all cell types and indicate that this wiring is specific to cell type.

The unique requirement for cyclin D1 in Ras- and Neu-induced mammary tumorigenesis, demonstrated here, together with earlier observations that ablation of cyclin D1 had virtually no consequences on adult mice physiology[9,10], suggests that anti-cyclin D1 therapy might be highly selective in shutting off the growth of human breast cancers while sparing other tissues. About 50% of human breast cancers contain amplification and/or overexpression of the c-*neu* (*c-erbB-2*, *HER-2*) gene[30]. Although mutations within the *ras* gene in human breast tumours are quite rare, several oncogenic pathways—including those originating from c-Neu/c-ErbB-2 and other receptor tyrosine kinases—were shown to signal through the Ras protein[20]. Our work suggests that human breast cancers with activated Neu–Ras pathways are prime candidates for anti-cyclin D1 therapy. □

Methods

Mice

MMTV-v-Ha-*ras* (ref. 11), MMTV-c-*neu* (strain TG.NK, ref. 12) and MMTV-c-*myc* (ref. 13) mice were purchased from the Charles River Laboratories; MMTV-*Wnt-1* mice[14] were purchased from the Jackson Laboratory. Transgenic mice were crossed with *cyclin D1*$^{+/-}$ animals. The resulting *cyclin D1*$^{+/-}$/MMTV-oncogene males were back crossed with *cyclin D1*$^{+/-}$ females yielding all four experimental groups: *cyclin D1*$^{+/+}$/MMTV-oncogene, *cyclin D1*$^{-/-}$/MMTV-oncogene, non-transgenic *cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ mice. Similar crosses were performed with cyclin D2$^{+/-}$ and cyclin D3$^{+/-}$ mice. Only females were used for subsequent analyses. All females except for MMTV-c-*myc* mice were kept as virgins

throughout the entire observation period. *cyclin D1*$^{+/+}$/MMTV-c-*myc* and *cyclin D1*$^{-/-}$/MMTV-c-*myc* females underwent two or three pregnancies. Pups were removed immediately after birth, to minimize the impact of breast-feeding (*cyclin D1*$^{-/-}$ females do not nurse their pups[9,16]). Mice were monitored by palpation twice-weekly for tumours.

Whole mounts of mammary glands

Inguinal mammary glands were removed and whole mounts were prepared as described[26].

Transgene levels

Total RNA was prepared from mammary glands[26] and reverse transcribed using ProSTAR Kit (Stratagene). The resulting complementary DNA was subjected to 18–30 rounds of PCR amplification using transgene-specific primers: 5′-GCAACAGTCCTAACATTCAC-3′ and 5′-TCGACGAACAAAGCAAACAG-3′ in 1 × PCR buffer (Perkin Elmer) containing 3.0 mM $MgCl_2$. The amplification products (predicted size 365 base pairs) were resolved on agarose gels, transferred to MagnaGraph membranes (Osmonics) and probed with a radiolabelled, internal, transgene-specific oligonucleotide, 5′-GGAAAGTGAAGGA-TAAGTGA-3′. For a control we used RNA prepared from mammary glands of non-transgenic littermates, which showed no amplification signal. Each lane corresponded to pooled mammary glands collected from 4–6 animals of each genotype.

Northern blotting

Total RNA was isolated; 20 μg of RNA was resolved with 1% MOPS–formaldehyde gels, transferred to MagnaGraph membrane (Osmonics), and probed with radiolabelled riboprobes specific for mouse cyclin D1, D2 and D3, as described[26].

Western blotting

Protein lysates were prepared; 50–150 μg of proteins were separated with 10% SDS–PAGE and transferred to Immobilon-P membrane (Millipore). The immunoblots were probed with the M-20 antibody (Santa Cruz), which recognizes cyclin D1 and D2, and anti-cyclin D1 (H-295, Santa Cruz) and D3 (C-16, Santa Cruz). As secondary antibodies, peroxidase-conjugated IgG (Jackson ImmunoResearch) were used followed by enhanced chemiluminescence (ECL) detection (Amersham).

Mammary cell lines

SH1.1 and 13Ma1a mammary carcinoma cell lines provided by P. Leder were grown in DMEM plus 10% bovine serum. Mouse non-transformed mammary epithelial cell line HC11 (ref. 18) was cultured in RPMI 1640 plus 8% bovine serum, 10 μg ml^{-1} epidermal growth factor and 5 μg ml^{-1} insulin.

Cells and retroviral infections

Wild-type and *cyclin D1*$^{-/-}$ mouse fibroblasts were prepared from day 13.5 embryos, as described[26], and the 3T3 cells were established.

pBabe-puro-Ras-V12 retrovirus, encoding activated Ras, was provided by R. A. Weinberg. pBabe-puro-NeuT retrovirus, encoding activated rat *neu* cDNA containing Val→Glu substitution at amino acid 664, was created by subcloning the *SalI-HindIII* 4.6 kb fragment of the pSVNeuT plasmid[31] into pBabe-puro vector. Phoenix cells were transfected with pBabe-puro-Ras-V12 or pBabe-puro-NeuT or with an empty pBabe-puro vector with calcium phosphate precipitation. Two days after the transfection, Phoenix cell supernatants were used to infect *cyclin D1*$^{+/+}$ or *cyclin D1*$^{-/-}$ 3T3 cells. Cells were selected for six days in puromycin (4 μg ml^{-1}).

Soft agar and tumorigenicity assays

Soft agar assays were performed using standard procedures. Briefly, logarithmically growing cells (10^4 and 10^5) were plated as single-cell suspensions in 0.4% agarose in DMEM supplemented with 10% fetal bovine serum. Cultures were analysed and scored after 5–28 days. Each cell line was analysed in quadruplicate. For tumorigenicity assays, 1×10^6 and 5×10^6 cells, resuspended in 200 μl PBS, were injected subcutaneously into flanks of female 6–8-week-old CD-1 nude mice (Charles River Laboratories). Mice were monitored for tumour formation and were killed 21–28 days after the injection. Eight mice were used to evaluate the tumorigenicity of a given cell line.

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